

D₂O UPTAKE RATE IN TWO BRACKISH-WATER NEREID POLYCHAETES

RALPH I. SMITH

Department of Zoology, University of California, Berkeley 4, California

In the previous study (Smith, 1963) *Nereis (Neanthes) succinea* was shown to have a higher salt-loss rate than *Nereis diversicolor* and *N. limnicola*, a difference correlated with the salinity of the natural habitats of these species. Under the standard conditions of the above experiments (a one-hour exposure to glass-distilled or deionized water following adaptation to sea water of chlorosity 10 g. Cl/L.), it was observed that *N. succinea* swelled greatly, to over 150% of its initial weight, but *N. diversicolor* swelled only to 125%, and *N. limnicola* to about 110% of initial weight. It is evident that in *N. succinea* the net water influx through the body surface is high, but the foregoing observations do not prove that *N. diversicolor* and *N. limnicola* are really less permeable to water. Quite possibly the latter two species have the same inward permeability to water (water influx per unit of surface) as *N. succinea*, but are more efficient in controlling volume by disposing of excess water as urine. It must also be considered that, although *N. diversicolor* and *N. limnicola* seem to approximate the "surface rule" in respect to salt loss as a function of size, *N. succinea* deviates in such a way as to suggest a change in shape during growth, so that the surface/volume ratio does not decrease as much as might be expected with increase in body size. *N. succinea* develops large parapodial lobes, which may be responsible for this effect. If so, the greater water intake by *N. succinea* might be the result of a relatively great surface, rather than of a higher water-permeability than is found in *N. diversicolor* and *N. limnicola*.

On the other hand, it would seem reasonable for animals living in water of low salinity to have included reduced permeability to water among their physiological or morphological adaptations although, as has been clearly shown by Shaw (1959) in the case of certain fresh-water crustaceans, the attainment of a reduced permeability to salts is not necessarily accompanied by an equally great reduction in water permeability. Since no data seem to have been published on the water-permeability of polychaetes, apart from studies of osmotic water influx, it seemed desirable to make a preliminary study of the penetration rate of "heavy water" (deuterium oxide, D₂O) into *N. succinea* and fresh-water representative of *N. limnicola*, in order to gain a general picture of the difference, if any, in the water-permeability of these euryhaline polychaetes.

METHODS

The D₂O influx was compared both under conditions of no osmotic water influx, and under conditions of hyperosmotic regulation in which there was presumed to be an inward movement of water as a result of an osmotic gradient. The worms were adapted in the laboratory for a week or more to dilutions of sea

water having chlorosities of 260, 104, and 26 mM/L., hereafter referred to by their approximate values of 50%, 20%, and 5% sea water and, in the case of *N. limnicola*, to fresh water from Lake Merced in San Francisco.

Five per cent D₂O mixtures from a 93.5 mole % stock were made up in the above media for test purposes, producing a slight lowering of their salt concentration, considered negligible in terms of osmotic stress for the euryhaline species used. Worms in 50% sea water are considered to be in osmotic equilibrium with the medium but those in 20% sea water and lower concentrations are known to be regulating, and thus maintaining an osmotic gradient which presumably causes a net inward water flux through the integument. After preliminary experiments had indicated that D₂O entrance in the absence of an osmotic gradient was very rapid (half-saturation times of less than five minutes), observations were confined to five-minute exposures to 5% D₂O, in order to reduce the complications of back-diffusion of D₂O and recycling *via* the urine. Worms were blotted dry on filter paper, weighed to the nearest mg. on a torsion balance, and dropped into 10–20-ml. volumes of 5% D₂O for five minutes. Worms were removed, quickly rinsed in a small strainer by a jet from a wash-bottle, blotted, and a sample of a few drops of coelomic fluid taken in a capillary pipette for estimation of the percentage of D₂O present. Most worms of less than 100 mg. wet weight were, however, sampled entire.

For determination of D₂O concentrations, sample drops (or whole worms) were distilled in "Aloc" disposable pipettes (short style) that had been boiled in distilled water and oven-dried. A light plug of cotton was inserted past the constriction, and the sample pipetted into the space between constriction and plug, the pipette base closed by a small cork, and the tip quickly sealed in a flame. For distillation the sealed pipettes were laid on a slide-warmer set at *ca.* 60° C., and covered with a folded towel. Their tips rested on the surface of a brass block set in a pan of ice and water. Distillation was allowed to proceed to dryness overnight, so that all H₂O and D₂O of the sample were transferred to the distillate. The use of only moderate heat lessened the chance of distilling over volatile contaminants, and controls indicated that drops of known D₂ concentration could be distilled in this way with negligible change of concentration.

The D₂O concentrations of the distillates were estimated by comparison with standards made up in distilled water and introduced into a bromobenzene-kerosene density-gradient column, the method being simplified after that introduced by Linderstrøm-Lang *et al.* (1938a, 1938b). I am indebted to Dr. Grover C. Stephens for suggesting the method and for advice on preparing the gradient. Details have been prepared for use in the author's class laboratory and will be furnished upon request of anyone interested. In its unrefined form, as used here, the method has proved adequate for obtaining what appear to be significant, although not very precise, data.

OBSERVATIONS

(a) *Comparison of rates of salt loss as a function of body size.* Data on salt-loss rates in the two species used in these D₂O uptake studies are given in the previous paper (Smith, 1963), but for comparison certain data are here replotted on a weight-specific basis. In Figure 1 are shown the rates of salt-loss (in mM/mg.

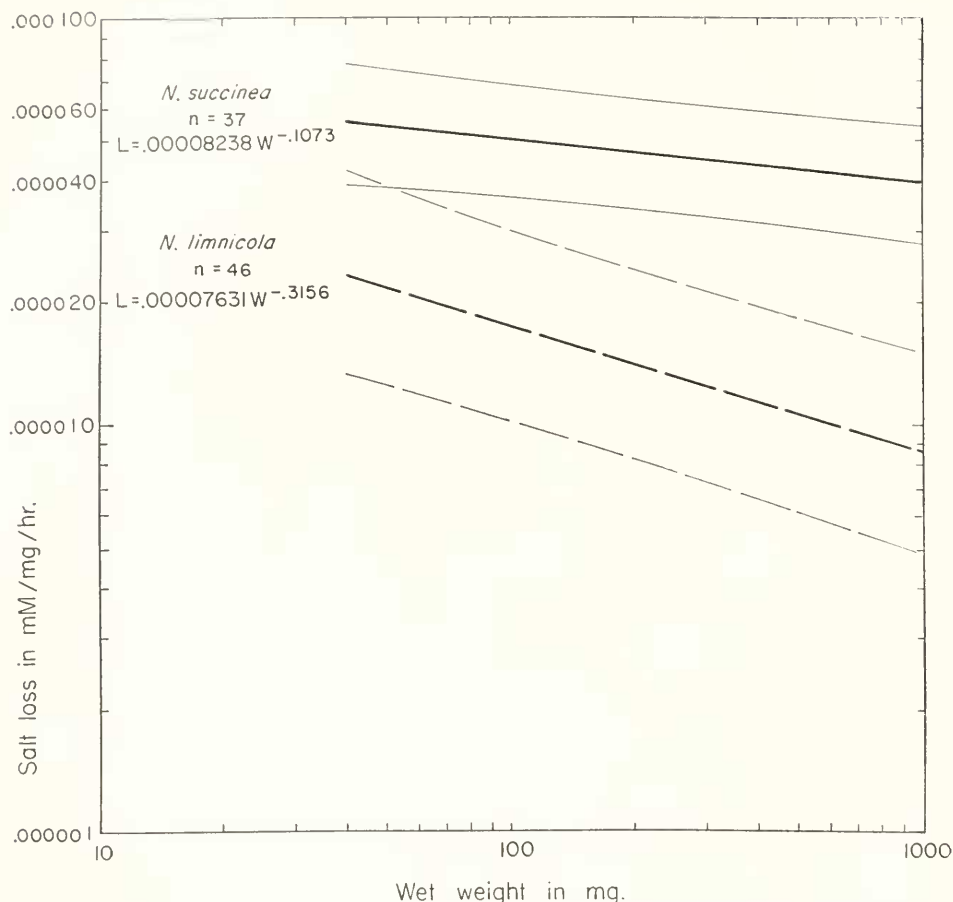


FIGURE 1. Weight-specific curves of salt-loss rate in *N. succinea* and *N. limnicola* as determined in a standard exposure to distilled water after adaptation in approximately 50% sea water; 95% confidence limits are drawn for each curve.

wet weight/hour) of worms adapted to 50% sea water and exposed for one hour in glass-distilled water. These curves are weight-specific salt-loss rate curves according to the expression:

$$L = \frac{\text{mM salt-loss/hour}}{\text{mg. wet weight}} = a (\text{Wet Weight})^{(b-1)}$$

For *N. succinea*: $L = 0.00008238 W^{(-0.1073)}$

For *N. limnicola*: $L = 0.00007631 W^{(-0.3156)}$

The slopes of these curves are significantly different ("t" test at 0.01 level). The slope of the curve for *N. limnicola* does not differ significantly from that characteristic of the "surface rule" ($b - 1 = -0.3333$). That of *N. succinea* deviates significantly from the surface rule; this is considered a consequence of the exten-

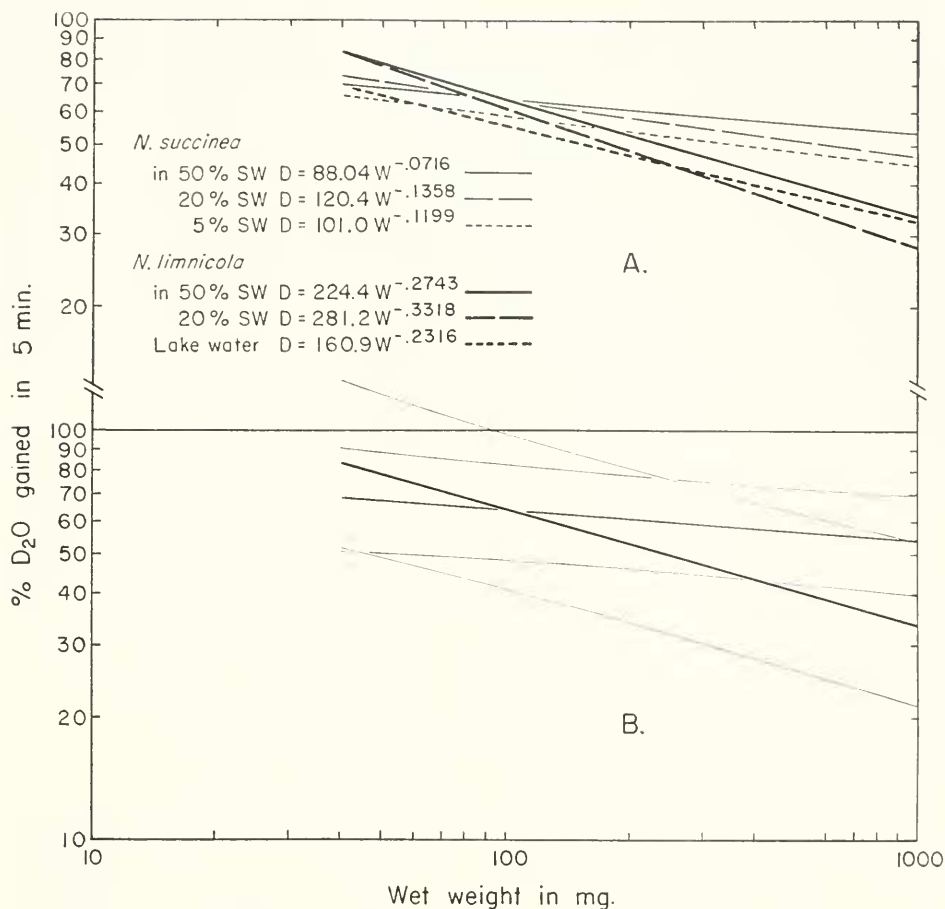


FIGURE 2. Weight-specific curves of rate of D₂O entry into *N. limnicola* and *N. succinea* in a 5-minute exposure (for details see text). Lines span the range of sizes used in the experiment. A. Curves for both species in ca. 50% and 20% sea water, as well as 5% sea water for *N. succinea* and lake water for *N. limnicola*. B. Curves for ca. 50% sea water only, replotted with 95% confidence limits drawn in.

sive flat parapodial lobes developed on older and larger members of this species, which tend to prevent much decrease in surface/volume ratio with increase of size.

The 95% confidence limits for the two salt-loss curves overlap for worms of less than 50 mg. wet weight, but over practically the whole range in which it has been possible to make such studies, it is clear that *N. succinea* loses salts at a much higher rate than *N. limnicola*.

b) *Comparison of rates of D₂O penetration as a function of body size.* In Figure 2 are shown curves for D₂O penetration in a five-minute exposure, expressing the concentration of D₂O in the coelomic fluid as a percentage of the D₂O concentration of the external medium. Several facts are apparent upon inspection of Figure 2:

(1) There is no difference in the penetration rate of D_2O into these two species, at least in the lower size range. Unlike the widely separated curves for salt-loss of *N. succinea* and *N. limnicola*, the D_2O penetration curves intersect within the size range of animals used experimentally. The permeability to D_2O is not correlated with the salt-loss tendency in these two species.

(2) It is equally clear that the groups of curves for the two species differ in slope in a characteristic way. Like the curve for salt-loss, the D_2O -penetration curves for *N. limnicola* have slopes approximating that predicted from the "surface rule"; those of *N. succinea* depart from this rule in the same sense as does the curve representing its salt-loss rate. For each species the slopes of the curves for salt-loss and D_2O -penetration do not differ significantly (t-test). Since D_2O -penetration is almost certainly *via* the body surface, the close correspondence in slope suggests that salt-loss in these two species is also, or in large part, a surface phenomenon.

(3) There is no suggestion that D_2O is entering these worms any more rapidly along an osmotic gradient than in the absence of such a gradient. In fact, the curves (Fig. 2A) for D_2O entrance in worms regulating against 20% sea water or lower concentrations suggest less entrance of D_2O . However, until a refinement of methods is achieved, it would be correct to say only that no increased entry of D_2O with an increase in osmotic gradient has been demonstrated. That the entry of D_2O may be less in lower salt concentrations is an interesting possibility, which would mean a lower water-permeability in such situations. Jørgensen and Dales (1957) have, indeed, suggested that this may be true in *Nereis diversicolor*.

(4) Figure 2B shows the extensive overlap of the 95% confidence limits for D_2O penetration rate in 50% sea water, in marked contrast to the lack of overlap for such limits in the case of salt-loss (Fig. 1). On this basis we may say that over the ordinary size range *N. succinea* shows a higher salt-loss rate than does *N. limnicola*, although these two species are equally "open" to the inward diffusion of D_2O , with or without an osmotic gradient. Although the slopes seem explicable in terms of the shapes of the worms involved, it is clear that the two species can be said to show an equal water influx at a wet weight of 100 mg. Extrapolation to smaller weights would be unreasonable. Upward extrapolation leads to an unrealizable prediction. For instance, the curves in Figure 2B intersect at a wet weight of 100 mg. and the 95% confidence limits if projected would not cease to overlap until a body weight of over 10 grams was reached (an academic point, since *N. limnicola* rarely exceeds one gram, and *N. succinea*, two grams). One can say that the deuterium oxide influx is the same in smaller members of both species, and that *N. limnicola* shows a decreasing water influx with size as a consequence of its falling surface/volume ratio. As in the case of salt-loss rates, *N. succinea* departs from this surface relationship, apparently by a change of shape during growth. The tendency of larger individuals of *N. succinea* to show a higher D_2O -influx than *N. limnicola* may be a consequence of relative surface area rather than the result of a higher water-permeability in the former species.

DISCUSSION

Although *N. limnicola* takes up less water than *N. succinea* when subjected to an equal osmotic gradient, the above observations show that both species are very

"open" and about equally permeable to the inward diffusion of D₂O even in the absence of an osmotic gradient or net inward water flux. The fact that *N. limnicola* takes advantage, so to speak, of the surface rule means that large individuals of this species would take in less D₂O than equivalent specimens of *N. succinea* but, as has been pointed out, this difference is essentially only a morphological one. From the physiological point of view, the most interesting differences are the discrepancy between the observed osmotic water intake and that calculated from D₂O diffusion, and the discrepancy between the extents of osmotic swelling shown by the two species. The present data do not permit a complete treatment of this problem, but the following preliminary considerations will indicate some of the problems that must be solved in consequence of the complex nature of the "permeability" of the brackish-water nereids.

For purposes of discussion, assume that in the approximately 50% sea water used in these experiments the worms are not regulating and that the water concentration both inside and outside the worms is the same. In this osmotic equilibrium there should be an equal diffusion of water inwards and outwards, that is, there should be no net water flux in either direction. Now, in this equilibrium situation, 100-mg. specimens of both *N. limnicola* and *N. succinea* have been shown to have the same D₂O uptake, namely, 64% of the external D₂O concentration is reached by inward diffusion in five minutes (1/12 hour). Ignoring back-diffusion and other complications, and accepting a water content value of 85% of the wet weight of the worms (an approximation based on unpublished data of Mr. Larry C. Oglesby of this laboratory), and assuming that D₂O were to replace all H₂O in the external medium, the D₂O diffusional influx may be calculated as $0.64 \times 85 \times 12 = 653$ mg. of water (as D₂O) entering per hour, by diffusion. In such an hypothetical situation, the external concentration of D₂O would be 49.4 osmolar. The inward D₂O diffusion per osmole of concentration difference would be (653 mg. per hour/49.4 osmoles) = 13.2 mg. D₂O per osmole of concentration difference per hour, and this (since we are assuming that D₂O is equivalent to H₂O) would be balanced by an equal and outward diffusion of water.

If we now consider the situation of a worm pre-adapted to 50% sea water and then placed into distilled water, the water concentration inside would still be 54.7 osmolar, and that outside would be 55.4 osmolar (pure (H₂O), a concentration difference of 0.7 osmole. Associated with such an excess concentration of water outside, there should be a net inward (osmotic) flow superimposed on the diffusional exchange. If the osmotic inflow were simply diffusional, and if we accept the diffusional influx as being 13.2 mg./osmole/hour, the net inward (or osmotic) inflow is 0.7×13.2 mg./hour = 9.24 mg. hour. The figure of 9.24 mg. equals 10.8% of the body water of a 100-mg. worm, and this should represent the gain in weight taking place in one hour in distilled water, which was the standardized osmotic emergency used in the previous study (Smith, 1963). But, it is clear that the observed osmotic swelling in *N. succinea* was much greater than predicted on this basis of the D₂O diffusion rate. The agreement is, however, good in the case of *N. limnicola*:

	Observed increase in weight (% wet weight)	Observed increase as % of body water
<i>N. limnicola</i>	10%	11.8%
<i>N. succinea</i>	50%	58.8%

In view of the scatter of the data, the similarity of predicted and observed water intake of *N. limnicola* need not be stressed. But there is remarkable disagreement between predicted and observed water intake in *N. succinea*, and equally remarkable is the difference in water intake between the two species in view of their equal influxes of D_2O .

That permeability, as measured by D_2O penetration, may be quite different from that estimated by osmotic water uptake, was first observed in the frog by Hevesy, Hofer and Krogh (1935), and has more recently been studied in vertebrate epithelia and eggs by several workers, including Koefoed-Johnsen and Ussing (1953), Prescott and Zeuthen (1953), Hays and Leaf (1962). Ussing (1954) offered as an explanation the concept of a surface combining areas through which diffusion might occur, and "pores" (or areas behaving like pores) through which a bulk flow of water might take place. The present data on D_2O uptake suggest that *N. limnicola* and *N. succinea* offer equally open diffusional pathways for water entry. If this view can be accepted, then either or both of two other factors may explain the observed differences in the degree of osmotic swelling undergone by the two species.

The first possibility is that these species differ in "porosity," with *N. limnicola* having such small "pores" as to impede bulk flow of water. The second possibility is that the two species do not differ at all in "porosity" (or in the tendency to permit osmotic water entry), but differ only in their ability to eliminate excess water as urine. On the latter hypothesis, the observed difference in net or osmotic water uptake as shown by swelling would be accounted for by the expulsion as urine by a 100-mg. *N. limnicola* of 58.8–11.8%, or 47%, of 85 mg. = 40 mg. of urinary water per hour *more than* that expelled by *N. succinea*. This value of 40% of the body weight in urine per hour is high, but is based upon the conditions of an osmotic emergency plus the assumption of equal "porosity" in the two species. It would be at least as reasonable to suppose that both porosity and urinary output differences are concerned as to assume one in the absence of the other. Even if *N. limnicola* could be shown to have an output of urine as high as 40% of the body weight per hour in the stated situation, its output in the natural fresh-water habitat would not be above 20%, since in that situation its internal concentration would not approximate that of 50% sea water, but would be closer to that of 25% sea water. But, until an absolute value for the urinary output of *N. succinea* is available, the volume of urine actually produced by *N. limnicola* cannot be estimated from the present data.

The observed equality of D_2O -influx, independent of the apparent rate of osmotic water intake, suggests that *N. limnicola* has the more effective water-elimination system, which would be consistent with its demonstrably better volume control. It is further evident that the D_2O influx bears no relation to the tendency to outward passage of salts. These facts need to be considered in future studies on osmoregulation in polychaetes; their full significance must await further experimentation. At present the actual water-permeability coefficient of these nereids cannot be estimated, since we do not know the surface area of these complexly-shaped animals, nor can we assume that all parts of their surfaces are equally permeable. That vertebrate epithelia represent complex systems for penetration by water has been repeatedly indicated (references cited above), in studies

that show a clear separability of diffusion permeability and osmotic (filtration) permeability. This distinction has not previously been stressed in studies on invertebrates, in which water-permeability has usually been estimated from osmotic fluxes.

SUMMARY

1. A simplified method for the estimation of heavy water (D₂O) uptake in polychaetes is presented.

2. It is shown that, although *Nereis limnicola* shows a smaller salt-loss and less net osmotic water uptake in a salt-free medium than does *N. succinea*, both species show an equal D₂O influx at a body weight of ca. 100 mg.

3. As in the case of salt-loss, *N. limnicola* follows the "surface rule" in its D₂O uptake; *N. succinea* does not.

4. No increased uptake of D₂O has been demonstrated in lower salinities in which there is presumed to be an osmotic inflow of water. This is consistent with the possibility that water-permeability is less at low environmental salinities.

5. A preliminary analysis of the osmotic and diffusional influxes of water in these two species suggests that their different osmoregulatory capabilities may be related to urine output (greater in *N. limnicola*), to a difference in integumental "pore size" (smaller in *N. limnicola*), or to some combination of these factors.

LITERATURE CITED

- HAYS, R. M., AND A. LEAF, 1962. Studies on the movement of water through the isolated toad bladder and its modification by vasopressin. *J. Gen. Physiol.*, **45**: 905-919.
- HEVESY, G. V., E. HOFER AND A. KROGH, 1935. The permeability of the skin of frogs to water as determined by D₂O and H₂O. *Skand. Arch. Physiol.*, **72**: 199-214.
- JØRGENSEN, C. B., AND R. P. DALES, 1957. The regulation of volume and osmotic regulation in some nereid polychaetes. *Physiol. Comp. et Oecol.*, **4**: 357-374.
- KØEFOED-JOHNSEN, V., AND H. H. USSING, 1953. The contributions of diffusion and flow to the passage of D₂O through living membranes. Effect of neurohypophyseal hormone on isolated anuran skin. *Acta Physiol. Scand.*, **28**: 60-76.
- LINDERSTRØM-LANG, K., O. JACOBSEN AND G. JOHANSEN, 1938a. On the measurement of the deuterium content in mixtures of H₂O and D₂O. *C. R. Lab. Carlsberg, Sér. Chim.*, **23**: 17-25.
- LINDERSTRØM-LANG, K., AND H. LANZ, JR., 1938b. Studies on enzymatic histochemistry. XXIX. Dilatometric micro-estimation of peptidase activity. *C. R. Lab. Carlsberg, Sér. Chim.*, **21**: 315-388.
- PRESCOTT, D. M., AND E. ZEUTHEN, 1953. Comparison of water diffusion and water filtration across cell surfaces. *Acta Physiol. Scand.*, **28**: 77-94.
- SHAW, J., 1959. Salt and water balance in the East African fresh-water crab, *Potamon niloticus* (M. Edw.). *J. Exp. Biol.*, **36**: 157-176.
- SMITH, R. I., 1963. A comparison of salt loss rate in three species of brackish-water nereid polychaetes. *Biol. Bull.*, **125**: 332-343.
- USSING, H. H., 1954. Membrane structure as revealed by permeability studies. Pp. 33-42 in "Recent Developments in Cell Physiology," Proc. Seventh Symp. Colston Res. Soc., Academic Press, New York.

